Preparative HPLC of Carbohydrates

INTRODUCTION

For the purpose of this chapter, preparative high performance liquid chromatography (HPLC) is defined as HPLC for the specific purpose of isolating pure carbohydrates for subsequent use. The intended use dictates the scale of the chromatographic equipment and the amount of carbohydrate to be isolated. Hence, isolation of a few nanograms of a biologically active oligosaccharide or of several kilograms of an experimental carbohydrate-derived bulking agent each qualify as preparative HPLC by this definition.

The conditions found in many of the HPLC methods described in this book — direct injection of samples without need for pre-column derivatization, high-resolution separations and nondestructive detection — are all ideal for preparative applications. This chapter will cover additional information that applies specifically to the practical use of preparative HPLC for carbohydrates. This information should be of value to those attempting to use preparative HPLC for the first time and should also provide some recent information for the more experienced chromatographer. Reviews have previously been published on general preparative HPLC methods [1–3] and on specific techniques for small and large scale preparative HPLC of carbohydrates [4]. The reader is referred to these works for detailed information, especially about published methods prior to 1986.

9.2 EQUIPMENT

Optimal preparative HPLC occurs when the desired amount of a pure compound is isolated in the shortest amount of time, while using the least amount of resources. Often, small high-resolution equipment will be more efficient than a large, low-

resolution system that produces impure, dilute fractions that must be concentrated and re-chromatographed. Some guidance on selection of equipment for preparative applications follows.

9.2.1 Solvent delivery systems

Preparative HPLC is often carried out on analytical-scale equipment, where only minor changes in hardware, such as installing a larger-volume injector loop, are required. Columns with lengths of 250–300 mm and inner diameters (i.d.) of 4–10 mm can usually be supported on such analytical solvent delivery systems if they are capable of producing flow rates up to 10 ml/min. For use of columns with i.d.'s of 20–40 mm and above, in which flow rates in the 25–50 ml/min may be required, solvent delivery systems with interchangeable, high flow-rate pump heads [5,6] are especially useful. Some of these systems, which can provide flow rates up to 200 ml/min or more, are limited in their maximum operating pressure. Fortunately, HPLC system pressure is more related to column length than width, so that a stationary phase that generates a system pressure of 1500 psi in a 5 mm i.d. column will usually develops a similar pressure in a 20 mm i.d. column of the same length when it is operated at the same linear flow velocity.

9.2.2 Pre- and guard columns

Pre-columns are highly recommended for preserving the "life" of analytical columns used in the preparative mode. Those that have replaceable cartridges and filters are highly recommended. Larger guard columns for use with 20 and 40 mm i.d. preparative columns are usually not practical because of their high cost and short life. Hence, thorough clean-up of samples prior to injection on the preparative column, is recommended. Preparative samples can be easily pre-treated by passage through a low pressure column (by gravity or suction) of the same or a similar type of stationary phase that is used in the preparative HPLC column. These sorbents can usually be purchased in less expensive, low-performance grades that are more suitable for this purpose.

9.2.3 Injection systems

Either universal (variable capacity) or fixed-loop injectors are suitable for preparative injections. For manual, preparative injections, regular fixed-loop injectors may be equipped with large volume sample loops (up to 20 ml are commercially available). Custom loops in the 0.1–1.0 ml size are readily made by using O.75 mm (0.030) i.d. stainless steel or PEEK tubing, cut to the appropriate length. For automated injections, a separate pump [5,7] may be used to directly inject the desired volume of sample onto the preparative column.

Column hardware

Preparative HPLC requires the repeated injection of relatively large samples onto the column's stationary phase. These rigorous conditions can easily lead to unwanted losses in column efficiency and capacity. Loss of capacity can occur if the bonded phase is degraded or is hydrolyzed from the matrix material of the stationary phase. Loss of efficiency can occur when the stationary phase matrix, such as silica gel, slowly dissolves in the mobile phase, leaving a void near the column inlet. Both of these common problems can be ameliorated by using hardware in which the stationary phase is held in a disposable cartridge. When the stationary phase has irreversibly lost its capacity, the cartridge is discarded but the holder and/or end fittings are recycled to reduce cost. Some cartridge-type columns also contain threaded inlet fittings that can be hand tightened to eliminate voids at the inlet. By tightening these fittings at appropriate intervals, poor column efficiency can often be restored to the original conditions. Such axial compression systems are available on columns up to about 40 × 250 mm, and they are useful for multi-gram scale isolations of carbohydrates [5,6]. The design of larger diameter columns for preparative liquid chromatography was recently reviewed [8]. The use of dynamic axial compression systems, in which a piston is used to maintain compression of the packed bed while the column is in operation, is discussed. These types of systems are useful for multi-kg scale separations. More sophisticated moving-bed and simulated moving-bed systems have been designed for continuous, industrial scale separations of carbohydrates [3,9,10].

Some stationary phases are relatively easy to pack into preparative columns by the user. The preparation and packing of polystyrene-divinylbenzene-based ion-exchange phases and their use for the separation of mono- and oligosaccharides has been described [5,11–14]. Equipment for packing analytical and preparative columns is commercially available [5,14–18].

9.2.5 Detection systems

Refractive index (RI) detectors are quite useful for preparative applications [5,6]. Since they are "mass sensitive" detectors, they provide a fairly accurate picture of the relative amounts of each component in a mixture. More selective detectors, such as the UV-types, detect compounds on the basis of a specific molecular property, such as the molar extinction coefficient. Selective detectors may be useful for determining where, in the chromatogram, one should collect fractions for a desired component. They may not, however, reveal the presence of major non-UV absorbing components that may co-elute with the compound of interest. This same reasoning holds for other selective detectors, such as those based upon fluorescence or electrochemistry. This fact should be kept in mind if the user plans to use one of the selective detectors described below.

A serious deficiency in RI detectors is that they can only be used to monitor isocratic separations and not gradient separations which are frequently required to separate complex mixtures of closely related oligosaccharides.

UV detectors may be used for detection of certain carbohydrates, such as neutral oligosaccharides isolated from glycoproteins after hydrazinolysis and re-*N*-acetylation [19]. Unlike RI detection, UV detection at low wavelengths may be used to monitor gradient separations of complex oligosaccharides [20].

When the carbohydrate of interest does not contain acetyl- or other UV absorbing chromophores, direct UV detection may not be possible. To address this issue, several methods have been recently published to "reversibly" derivatize oligosaccharides to provide temporary UV chromophores. This technique enables the researcher to conduct gradient separations, monitored by UV detection, and to isolate pure derivatives that can be subsequently converted back into the original reducing oligosaccharide (which is frequently needed for biological or chemical assays). For instance, Mort et al. [21,22] added UV absorbing groups to oligogalacturonic acids and malto-oligosaccharides, enabling gradient elution and preparative isolation of the former compounds. The chromophoric aminopyridine group, attached by a glycosylamino-linkage was readily hydrolyzed to yield the original oligosaccharide. Although such reactions could lead to degradation of the oligosaccharides through Amadori rearrangements, relatively high recoveries of the parent oligosaccharides were reported. Kallin et al. [23] used *N*-fluorenylmethoxycarbonyl (FMOC) glycosylamine derivatives of human milk- and other oligosaccharides, which, after chromatographic isolation, were converted back into the original oligosaccharides in yields of about 90%.

Pulsed amperometric detectors (PAD's) are now frequently used for detection of carbohydrates after analytical high performance anion-exchange chromatography (HPAEC). Because such detection results in the oxidation of carbohydrates, it has frequently been assumed that this seemingly destructive detection method would not be appropriate for monitoring preparative chromatograms. These concerns have been addressed in a recent review [24] where it was noted that under most conditions, only about 1% of the carbohydrate eluting from the column actually is oxidized by the detector. For preparative applications, where large amounts of carbohydrate are being fractionated, this percentage is probably less. This information seems to be verified in many studies (for instance, see [25,26]) in which PAD has been used for this application without significant loss of compounds.

In many respects, mass spectrometers represent the ultimate HPLC detector, and direct coupling of HPLC systems to mass spectrometers can preclude time consuming intermediate preparative purification steps. Many advances have been made on the direct coupling of mass spectrometers to HPLC systems. Direct coupling of reversed- or normal phase systems to mass spectrometers has been used for some time for the direct analysis of carbohydrates [4]. The coupling of HPAEC systems, which frequently use non-volatile buffer components and highly alkaline mobile phases, to mass spectrometers has been accomplished by the use of on-line

micromembrane supressors to remove unwanted, ionic mobile phase components. By this method, HPAEC systems have been coupled to mass spectrometers through a variety of interfaces (see Section 9.2.7 and Chapter 5).

9.2.6 Fraction collectors

For the automated, unattended repetitive injection and fractionation of carbohydrate samples, a microprocessor-controlled "intelligent" fraction collector is required. Various models are commercially available and they have been used for collection of samples over 24 hour unattended periods [5,7].

9.2.7 Miscellaneous equipment

Commercial micromembrane suppressor units can be useful for the on-line neutralization of certain high-pH eluents from HPAEC systems. Purified carbohydrates contained in these eluents are thereby protected from alkaline degradation reactions that could occur upon extended contact with a highly basic mobile phase. It has been reported [27] that such systems can neutralize and remove sodium ions from column effluents containing up to 0.2 M NaOH. The resulting deionized solutions had pH values of 5.5–6.0 and there were minor losses (<10%) of neutral oligosaccharides and alditols. It is important to note that these results were achieved with analytically-sized (4 mm i.d.) columns at flow rates of about 1 ml/min. The presently available micromembrane units are not capable of neutralizing eluents at the higher flow rates required for 10 and 20 mm i.d. columns. In these larger scale cases, fractions must be manually neutralized by the addition of dilute acids or hydrogen-form cation-exchange resins. Anionic micromembrane suppressors have been used to remove sodium ions from HPAEC eluents so that direct coupling to mass spectrometers through on-line thermospray [28,29], ion-spray [30] and particle beam interfaces [31] can be achieved. Anionic micromembrane suppressors do not, of course, remove anionic species such as acetate, formate or phosphate from the column effluent. In those cases, the suppressor would exchange sodium or other cations present for protons, producing the free acid forms of those species. Removal of such acids can easily be done by treatment of the solution with anion-exchange resins. However, when the carbohydrate to be isolated is also anionic in character such as in the case of low molecular-weight sialic- or oligogalacturonic acids, removal of buffer anions from isolated fractions is a difficult challenge. One report [32] claimed that a commercial device was capable of concentrating and desalting (removal of imidazole–hydrochloric acid buffer) oligogalacturonic acids that were purified by low-pressure preparative anion-exchange chromatography. The device, called a "Microacilyzer S1" automatic desalting device, operates with ion-exchange membranes.

Rapid desalting and filtering of larger oligosaccharides (>1900 Da) has been conveniently accomplished by a method called centrifugal size-exclusion chromatography [33] in which a small quantity of sample is forced through a microcolumn of

size-exclusion gel by use of common laboratory equipment and centrifuges. Since several samples can be done simultaneously, the amount of time required per sample is negligible.

9.3 STATIONARY PHASES

9.3.1 Normal phases

Normal stationary phases, of which there are many types, are relatively polar and are usually eluted with aqueous mixtures of water and an organic solvent, such as acetonitrile. These phases are described as "normal" phases because increasing the polarity of the mobile phase (increasing the percentage of water) results in lower retention and faster elution of polar carbohydrates from the column. The historical development and recent applications of these phases have been reviewed [4,34].

Aminoalkylated silica gels, like all normal phases (also called polar sorbents, see Chapter 3) are very useful for the preparative separation of neutral sugars and oligosaccharides since they provide high resolution separations and they have the highest capacity [5,35] of any of the commonly used stationary phases. When they are used in conjunction with higher percentages (>75–80%) of acetonitrile in the mobile phase, many individual mono- [35], di- [36] and tri-saccharides [37] can be resolved. Mobile phases with lower amounts of acetonitrile (50–60%) are used for class fractionation of many oligosaccharides in ascending order up to dp 8 or higher [6]. Despite the many advantages of this type of phase, there are some disadvantages that must be considered. First, aminoalkylated silica gels are known to dissolve in water-rich mobile phases, leaving voids in the inlet end. These voids can be removed, to a certain extent, by using columns equipped with a dynamic axial compression inlet fitting, as described in Section 9.2.4. Otherwise, one can delay this process, by placing a second column packed with silica gel between the pump and injector, to saturate the mobile phase with soluble silica gel. Regardless of whether this silica gel "saturator" column is used or not, collected fractions will contain leached silica gel, which may be difficult to remove.

The formation of in situ generated Schiff bases and glycosylamines between reducing sugars and stationary-phase amino groups [38–41] is an often overlooked process that leads to poor sample recovery (as low as 10% in some cases) for reducing sugars such as arabinose, ribose, galactose, mannose, xylose, rhamnose and oligosaccharides with those sugars on the reducing end. Not only does this process cause poor recovery of carbohydrates, it also alters the selectivity of the stationary phase. Buffering the mobile phase with phosphate buffer [38] can minimize these interactions but this necessitates an additional step for removing these buffer components from the purified carbohydrate fractions.

Many oligosaccharides, such as cellodextrins and larger (>dp 8) malto-oligosaccharides, are either insoluble or only sparingly soluble in acetonitrile/water mobile phases. This limits the amount of oligosaccharide that can be injected and isolated in each preparative HPLC run. Under these conditions, large amounts of (expensive)

acetonitrile may be required for isolation of a few milligrams of oligosaccharide and hence, for these cases, other chromatographic phases (see below) may be more appropriate.

Excellent analytical separations of sugars and oligosaccharides have been reported [35] on un-modified silica gel stationary phases, when various amino compounds are added to the mobile phase. This in-situ-generated amine-modified silica gel system is not recommended for preparative HPLC of the reducing carbohydrates listed above, because of their known reactivity with amines.

Recently, new experimental [42] and commercially available [43,44] polar amino-phases have been developed and they are reported to be very stable after extensive use for carbohydrate separations. The phase used in the latter two examples is based on a vinyl alcohol copolymer gel, rather than a silica gel, and is reported to be stable over a wide pH range (2–11). Because of the stability and the absence of leachable silica gel, fractions collected after preparative HPLC on this phase should be relatively free from the impurities that usually originate from the more traditional normal phases described above.

"Diol"- [40] and "polyol"-bonded [45] silica gels have been used as alternative normal phases for analytical HPLC of carbohydrates and have characteristics that make them good candidates for preparative use. The mobile phases required and the selectivity exhibited by these phases are both similar to that of aminoalkylated silica gels but, since no amino-groups are present on these phases, the chromatographer need not worry about the formation of Schiff bases with reducing sugars (see above). Efficiency and resolution of reducing carbohydrates is generally not as high as on aminoalkyl phases, partly because stationary-phase amino groups catalyze the rapid interconversion of anomeric forms of carbohydrates. Without these catalytic groups present, mutarotation of many carbohydrates is slow, relative to the time of the chromatographic run and the individual anomeric forms are partially or fully resolved (see [4] for a review). Hence, chromatographic peaks on diol-bonded phases may be broader or more complex to interpret than those on amino-type phases.

Cyclodextrin-bonded phases are a type of normal phase that has recently been used for the analytical HPLC of sugars [46] and oligosaccharides [46,47]. This phase has similar selectivity to amino-type phases and appears to be exceptionally durable over extensive periods of use. Neutral carbohydrates are readily eluted from this column in the normal phase mode but acidic carbohydrates are strongly retained on this phase, as they would be on a weak anion-exchange column. These features make this phase useful for separation of mixtures of acidic and neutral carbohydrates such as, for instance, lactose and lactobionic acid [48].

9.3.2 Reversed phases

Common phases of this type include the alkylated- (C_8 and C_{18}) and phenyl-bonded silica gels and the polystyrene/divinylbenzene copolymer gels, see Chapter 2. These phases are of limited use for fractionation of polar sugars and small oligosaccharides since these carbohydrates are poorly retained on these nonpolar

phases. Reversed phases are of more value for separation of less polar carbohydrates such as methyl glycosides [49], chitin- [50,51], starch- [5,52], human milk- [53] or glycoprotein-derived [54] oligosaccharides. For a more complete review of reversed-phase separations of carbohydrates, see [4] and Chapter 2.

Unlike normal phase columns, where partitioning occurs throughout the porous silica gel particle, partitioning of the analyte in reversed-phase systems occurs between the mobile phase and the small amount of non-polar, bonded "liquid" phase. For this reason, reversed-phase columns have relatively low capacities, especially for polar carbohydrates. As a matter of comparison, a 20 × 250 mm column packed with a normal, amino-type phase was capable of fractionating [5] 500 mg of malto-oligosaccharides per injection, while a similar size C_{18}-bonded phase was only capable of fractionating about 12 mg of this sample. Many reducing oligosaccharides are partially resolved into anomeric forms on reversed-phase columns, leading to broad, complex peaks (see above and [4] for a complete explanation). Such irregularity in peak shape leads to poor resolution and difficulties in manual and automated fraction collection. The silica gel-based reversed-phase columns are also reported [55] to be somewhat unstable when eluted with aqueous mobile phases that are used for carbohydrate separations.

Reversed-phase columns can be used in conjunction with ion-pairing reagents for the fractionation of acidic oligosaccharides [56], see Chapter 2. Addition of an alkylamine, which interacts both with the hydrophobic stationary phase and the anionic oligosaccharide, increases the retention and resolution of the acidic carbohydrates to be isolated. For preparative applications, the ion-pairing reagent must be non-reactive towards the carbohydrate and it should be easily removed from the final, purified fractions.

A special type of reversed-phase material is the porous graphitized carbon column recently used for the preparative isolation of *myo*-inositol-containing oligosaccharides [57] and for the isolation of neutral and acidic oligosaccharides, alditols and glycopeptides derived from glycoproteins and colostrum [58,59]. The capacity and durability of these columns are reported to be good and they have a different selectivity toward oligosaccharides and glycopeptides than typical alkylated silica gels. An advantage of this phase is that hydrophobic peptides, glycopeptides, reducing oligosaccharides, sialylated oligosaccharides and oligosaccharide alditols can be chromatographed under the same conditions. Carbohydrates isolated by this system (using an acetonitrile/dilute trifluoroacetic acid mobile phase) are free from non-volatile buffer components frequently used with HPAEC systems and the resulting chromatographic fractions can be evaporated and then directly analyzed by mass spectrometric techniques [59].

9.3.3 Cation-exchange resins

A recent survey [34] showed that cation-exchange resins are the most popular stationary phase among those who routinely conduct analytical HPLC of simple

carbohydrates, see Chapter 4. This is understandable since these phases are very durable and inexpensive to operate (mobile phase is usually pure water). These same properties make these phases very useful for preparative applications. The historical aspects and technical details of these phases have been reviewed earlier [4]. In summary, these phases are usually spherical, sulfonated gels made from polystyrene that is cross linked with divinylbenzene. Columns containing these phases are commercially available in a variety of metal-ion forms (Na^+, Ca^{++}, Ag^+, K^+, Pb^{++}), particle sizes (about 7–30 μm), and degrees of cross-linking (2–8%). The separation of neutral carbohydrates on these phases is based upon a combination of size exclusion and ligand exchange modes in which oligosaccharides are separated by size (with a descending order of elution) and then by ligand exchange interactions between the bound metal ions and the hydroxyl groups of carbohydrates. For a review of these mechanisms see [60], [4] and Chapter 4. Columns containing these phases must usually be operated at elevated temperature, up to 85°C, so that reducing carbohydrates will elute as narrow, symmetrical peaks. At lower temperatures, individual anomeric forms of sugars can be partially or fully resolved. This usually produces broad, multiple and overlapping peaks, which makes collection of pure fractions difficult.

The capacities of cation-exchange phases are relatively high (100 mg samples can be injected onto a 20 × 250 mm column [5]) and since no buffers or organic phases are usually required, isolated fraction can be evaporated and used without further treatment. Of special note is the durability of these columns. A preparative column containing Ca^{++}-form cation-exchange resin has been used in the author's laboratory for over 5 years without noticeable loss of capacity or resolution. Preparative columns (20 × 250 mm) of these resins are quite easily packed using published procedures [5,12–14] and they can be used on standard analytical HPLC systems since they operate at surprisingly low flow rates (1–2 ml/min) and pressures (100–500 psi).

A general guide for the selection of cation-exchange phase for particular applications was published previously [34]. In general, for isolation of neutral mono- and di-saccharides, 4–8% cross-linked resins in the Ca^{++} [14,61] or Pb^{++} form is recommended. For acidic mono- and di-saccharides, an 8% cross-linked resin in the H^+ form is recommended [14]. For separation of neutral oligosaccharides up to dp 7–8 [5], a 4% cross-linked resin should be used in the Ag^+ or Na^+ form. The use of H^+-form resins with only 2% of cross-linking allows separation of neutral and acidic oligosaccharides up to a dp value of 10–12 [62]; however, this low amount of cross linking results in a fragile phase that may not withstand rigorous preparative applications.

Some precautions must be taken when using cation-exchange resin phases. Fractions isolated from Ag^+-form columns may contain traces of silver ions, which can cause oxidation of reducing sugars. Collected fractions should be deionized with a mixed-bed ion-exchange resin prior to evaporation and storage of fractions. It is also important to note that some oligosaccharides are not stable under the high-temperature conditions described above. For instance, for isolation of sucrose,

ketoses and other inulin-type oligosaccharides containing labile fructofuranosyl linkages, one should not use a H^+-form resin at elevated temperature, since these conditions would result in hydrolysis of the oligosaccharides. Similarly, columns packed with Ag^+-form resins have the same capacity to hydrolyze these weak glycosidic linkages, since such resins are usually only converted about 75% into the Ag^+ form, with the remainder in the H^+ form [13]. Inulin-type oligosaccharides, however, can be separated well on H^+- and Ag^+-form resins at room temperature, where no hydrolysis occurs. This is possible because these oligosaccharides are not reducing sugars and therefore, are not separated into anomeric forms at low temperatures (see above). It has also been reported that 2-pentuloses and trioses [63] are unstable when chromatographed on heated Pb^{++}-form ion-exchange resins. Hence, use of these resins for isolation of those compounds is not recommended.

9.3.4 Anion-exchange phases

Amino-bonded silica gels function as weak anion exchangers when they are eluted with aqueous buffers or acetonitrile/aqueous buffer combinations. Advantages of these phases include high capacity and high selectivity. With this system [64], up to 400 mg of oligogalacturonic acids were readily fractionated on a 21.4 × 250 mm column to yield purified fractions of dp 2–6 oligomers. The major disadvantage of this phase lies in its gradual loss of selectivity and efficiency. The latter problem is remedied by the use of axial compression fittings on the column hardware [64].

Pellicular, high performance anion-exchange (HPAE) resins have recently become popular for analytical separations of neutral and acidic carbohydrates (see Chapter 5) and are also now being used for preparative applications. These phases provide extremely good resolution so that isomeric oligosaccharides with slightly different linkages can frequently be separated. For separation of neutral oligosaccharides, these phases are usually eluted with high-pH (11–12) mobile phases. Such conditions are necessary for ionization of sugar hydroxyl groups to produce the required anion that interacts with the positively charged stationary phase. It is well known that under alkaline conditions, reducing oligosaccharides can undergo peeling and isomerization reactions. This area has been thoroughly addressed in a recent review [24]. It appears that isomerization, β elimination or other degradative reactions are relatively insignificant during HPAEC unless the oligosaccharide's reducing terminus is a 2-acetamido-2-deoxy hexose, or if the reducing terminus is 3-*O*-substituted. In order to avoid problems in these two situations, one can use reversible, precolumn derivatization, as described in Section 9.2.5, prior to chromatography. Alternatively, the reducing terminus can be reduced to the alditol prior to chromatography. This latter process, of course, permanently alters the original structure of the oligosaccharide.

It is important to note that HPAEC phases can be used effectively at moderate pH values (5–8) for separation of acidic carbohydrates such as oligogalacturonic

acids [65]. Semi-preparative (10 mm i.d.) versions of these columns can be used to isolate mg quantities of these acidic oligosaccharides [25].

A disadvantage of HPAE resins for preparative applications lies in their inherently low capacity. These pellicular particles, which contain a thin layer of stationary phase attached to a solid spherical core, were purposely designed to have a low capacity in order to optimize chromatographic resolution. While gram quantities of carbohydrates cannot be fractionated on standard sized HPAEC columns, 0.1–10 mg samples of neutral [26,66] and acidic [25,67] oligosaccharides can be readily isolated on columns with diameters of either 4.6 or 9.0 mm.

Strong anion-exchange phases based on silica gels [68] or organic polymers [69] have been used for preparative isolation of acidic carbohydrates. These fully porous particles have larger capacities than pellicular resins.

9.3.5 Miscellaneous phases

High performance liquid affinity chromatography, HPLAC [70], has been used for extremely selective separations of complex oligosaccharides. In one variation of this method, monoclonal antibodies were raised to selected oligosaccharide antigens. These antibodies were then bound to concanavalin A which had been previously attached to high-performance silica gel matrices. Oligosaccharides recognized by the monoclonal antibody were separated as they were eluted through the column. Only small (ng) amounts of oligosaccharides can be chromatographed, but recovery is reported to be quantitative and for many bioassays, these quantities are sufficient.

HPLAC has also been achieved by use of lectins, covalently bound to high-performance silica gel supports [71]. *N*-Glycanase-released oligosaccharides were separated on supports containing leukoagglutinating phytohemagglutinin, concanavalin A, *Datura stramonium* agglutinin and *Vicia villosa* agglutinin. Although this technique was used for analytical purposes, the authors stated that the columns were extremely stable after repeated use and that "... both capacity and resolution could be readily increased by the use of longer columns".

9.4 GENERAL GUIDELINES FOR PREPARATIVE HPLC OF CARBOHYDRATES

When developing a strategy for preparative HPLC separation of a specific carbohydrate, at least three factors must be considered. The first is to choose a stationary and mobile phase combination that will provide a separation with adequate resolution between chromatographic peaks. The second factor is to decide upon the size of the column needed for the application. Finally, one must determine the chromatographic conditions, particularly the flow rate, needed to affect the desired separation on the preparative sized column so that run time and resolution are optimized.

Obtaining adequate resolution (R)

Resolution (R) values can be estimated by the following equation:

$$R = \frac{\Delta t}{t_w} \tag{9.1}$$

where Δt is the time between adjacent peaks, and t_w is the average peak width (in time units) for the two peaks. When two similar sized peaks are separated by R values of 1 or greater, each peak can be isolated with less than a 5% contamination from the other peak, producing isolated compounds with greater than about 95% purity [5]. Selection of a stationary/mobile phase combination that will provide resolution values at this level is desirable. When resolution values are significantly below 1, re-chromatography of collected peaks may be required to produce high purity carbohydrates.

9.4.2 Selection of column size

As stated in Section 9.3, the type of stationary phase used in an application has a major impact on the quantity of sample that can be isolated during a preparative chromatographic run. Normal phase columns have relatively high capacities and mg amounts of carbohydrates can easily be isolated on analytically sized columns of this type. HPAEC phases have much lower capacities and frequently only sub-mg quantities can be loaded onto them. Some guidelines on the approximate loading capacities of the commonly used stationary phases are given in Table 9.1. Resolution values will vary with the amount of sample loaded. Overloading columns will result

TABLE 9.1

APPROXIMATE LOADING CAPACITIES OF STATIONARY PHASES FOR SIMPLE MONO- AND OLIGOSACCHARIDES

Stationary phase	Column size (mm)	Mobile phase	Flow rate (ml/min)	Maximum loading capacity (estimated from [ref.])
Normal phase (aminoalkyl-, diol-, cyclodextrin-, etc.)	4.5 × 250	CH_3CN/H_2O	1	12–25 mg [35]
	10 × 250	CH_3CN/H_2O	4	50–100 mg [35]
	22 × 250	CH_3CN/H_2O	12	250–500 mg [5]
	41 × 250	CH_3CN/H_2O	40	1.0–1.75 g [6]
Cation-exchange resin phase (H^+ or metal ion form)	7.8 × 300	H_2O	0.6	5–20 mg [14]
	20 × 300	H_2O	2.0	50–200 mg [5,14]
Reversed-phase (C_{18}-, graphite, etc.)	10 × 100	H_2O	1.0	1–10 mg [52]
	22 × 250	H_2O	4.0	10–150 mg [5,51]
Pellicular HPAEC phase	9 × 250	NaOH/NaOAc	4.0	0.1–10 mg [26,66]

in broad peaks and poor resolution. Experience, coupled with trial and error, are usually required to determine the maximum sample one can load while retaining adequate R values. Once the loading capacity of an analytically sized column is known, it is relatively easy to estimate the column size required for larger applications because sample capacity is proportional to the cross sectional area of the column [72], that is, to d_c^2, where d_c is the column i.d. Hence, if acceptable R is obtained when a 2 mg sample of carbohydrate is injected onto a 4 mm i.d. column, the same R should be obtained with a 8 mg sample injected onto a 8 mm i.d. column with the same length and identical stationary phase.

9.4.3 Selection of column flow-rate

Once an acceptable separation has been developed on an analytical column and it has been determined that a larger column is required for efficient preparative HPLC, one must next estimate the optimum flow rate for the larger column. To maintain a given separation *time* after scale-up to a larger column, one may use the following equation [5]:

$$F_p = F_a \left[\frac{\text{i.d.}_p}{\text{i.d.}_a}\right]^2 \frac{L_p}{L_a} \tag{9.2}$$

where F_p is the flow rate for the larger bore (preparative) column, F_a is the flow-rate for the analytical-scale column, i.d._p and i.d._a refer to the inner diameters of the preparative and analytical columns, respectively, and L_p and L_a, refer to the respective column lengths. Assuming that exactly the same stationary and mobile phases are used in each column, use of this equation will predict flow rates that will produce very similar retention times and R values for components on larger bore columns.

9.5 SPECIFIC PREPARATIVE HPLC TECHNIQUES FOR VARIOUS CLASSES OF CARBOHYDRATES

9.5.1 Neutral monosaccharides and derivatives

Preparative HPLC can be readily used to purify monosaccharides and their simple derivatives from synthetic or natural matrices. These simple sugars are often isolated on normal phases such as aminoalkyl silica gels (see Section 9.3.1) eluted with acetonitrile/water mobile phases. These phases have high capacities [14,35] for simple sugars which are quite soluble in the acetonitrile-rich mobile phases. This type of phase should not be used for those commonly occurring sugars that can form covalent linkages with the amino-groups of the stationary phase (see Section 9.3.1). The most practical stationary phase for isolation of monosaccharides is usually a cation-exchange resin phase (see Section 9.3.3), which is very durable and eluted with pure water. These phases (see Section 9.3.3.), in various metal-ion forms,

have good capacity for monosaccharides and their selectivity can be optimized for a particular separation by choosing the proper metal-ion form and pore size. The calcium- and other divalent metal forms are used for the separation of glucose and fructose at the mg [14] to the multi-kg [3] scale. The lead form is recommended for the separation of monosaccharides found in hemicellulose hydrolyzates [73] but is not recommended for isolation of trioses and 2-pentuloses, which are said [63] to be unstable on this phase. Reversed phases (see Section 9.3.2) are of little value for simple monosaccharides that are poorly retained and resolved. Methyl- [74] and benzyl-glycosides [75], having more hydrophobic character, are well retained, resolved, and readily fractionated on reversed phases, when water is used as an eluent. Glycosides of gallic acid [76] and various anthocyanins [77,78] have been isolated on the same stationary phases, eluted with methanol/water mobile phases acidified with phosphoric or trifluoroacetic acid.

9.5.2 Acidic and basic monosaccharides and derivatives

Uronic and aldonic acids have been successfully fractionated on the 1–40 mg scale [14,15] on cation-exchange resins [see Section 9.3.3] in the H^+ form. These same systems are useful for fractionating acidic carbohydrates in the presence of *N*-acetylated monosaccharides. When using a cation-exchange resin in the H^+ form, a mobile phase consisting of water and a volatile acid, such as formic acid, is employed. Fractions from such columns can be evaporated completely to yield the dried, pure carbohydrate fraction.

Sugar phosphates, phytic acid, and inositol phosphates have been separated on a variety of phases on the analytical, but seldom on the preparative scale. Simple mixtures of a sugar phosphate and a neutral sugar can easily be accomplished on either cation-[79] or anion-exchange [80] phases (see Section 9.3.4). A number of phosphorylated sugars and glycolytic intermediates have been separated by reversed-phase ion-pairing systems [81–84] and some low-resolution separations of pentose, hexose and heptose mono- and di-phosphates have been achieved on strong anion-exchange silica gels [85–86]. All of these methods can be used for small scale preparative isolations of these phosphorylated compounds.

Inositol phosphates are currently under intensive study in biochemical systems and methods for their separation have recently been developed. These include separations on ion-pair, reversed-phase systems [84,87–89], pellicular HPAEC phases [90] and traditional high-performance anion-exchange phases [91–93]. It is expected that all these phases, especially the latter with their larger capacity, will be useful for preparative HPLC applications.

An recent study [94] compared 7 different HPLC stationary phases for the separation of sialic acids. While none of the systems were capable of separating all 19 of the sialic acids examined in the study, HPAEC, with sodium acetate buffers, provided the most useful overall selectivity, resolution, non-destructive conditions

related compounds, such as the deoxy-2-octulosonic acids, have been reviewed previously [4].

Simple amino sugars have been separated on HPAEC phases [95] which provide excellent separations but have marginal capacity for preparative applications. A 1-amino-1-deoxy-2-fructose Amadori product, formed by the reaction of glucose with lysine, was purified on a preparative C_8-type reversed-phase column, eluted isocratically with methanol/isopropanol/ammonium hydroxide mobile phase [96].

9.5.3 Neutral oligosaccharides

Preparative HPLC of neutral oligosaccharides has been accomplished on practically every type of HPLC stationary phases. Homo-oligosaccharides, those containing only one type of monosaccharide residue and linkage, are the simplest to fractionate. Cellodextrins have been isolated on cation-exchange resins (see Section 9.3.3) in the Ag^+ [97] and Ca^{++} [11,98] form and on reversed-phase columns (see Section 9.3.2) [52]. Gram quantities of cellodextrins [97] up to a dp of 8 were isolated from a Ag^+-form column, using a standard analytical HPLC system equipped with a 20 × 250 mm column (Fig. 9.1). The purity of these oligosaccharides averaged over 90%. Normal-phase (see Section 9.3.1) columns are not recommended for this fractionation since cellodextrins are relatively insoluble in acetonitrile/water mobile phases. Malto-oligosaccharides up to dp 12 can be isolated in mg to g quantities by preparative HPLC on normal [5,6] and cation-exchange [5] phases. Figure 9.2 shows a comparison of reversed phase, normal phase and cation-exchange resin columns for this separation. The amounts of pure oligosaccharides that could be isolated from one run on each of these columns varied considerably. Only 1–2 mg of purified oligosaccharide could be isolated from each run on the reversed phase column, whereas 10–20 mg and 50–100 mg of each oligosaccharide were isolated from each run on the cation-exchange column and the normal phase column, respectively. Purities of the malto-oligosaccharides isolated from the latter two columns ranged from 88–96%. Chitin oligosaccharides [51] have been isolated on reversed phase, cation-exchange phases (various ionic forms) and on aminoalkyl-silica gel phases (Figs. 9.3 and 9.4). Chitin oligosaccharides are more hydrophobic than malto-oligosaccharides and this has a predictable and measurable impact on loading capacities of the stationary phases; the loading capacity is higher on reversed-phase than on normal phase columns (compare Figs. 9.2, 9.3 and 9.4). This is the opposite to that found with the more hydrophilic malto-oligosaccharides. Isomalto-oligosaccharides have been isolated on reversed-phase [52] and normal phase [99] systems. Oligosaccharides derived from inulins have been isolated on reversed- [100, 101] and normal [100] phase columns.

Separation of hetero-oligosaccharides by preparative HPLC is somewhat more problematic than that of homo-oligosaccharides and, frequently, combinations of two or more modes of chromatography are needed for complete purification. Such is the case with branched fructans [102,103] which require sequential fractionation

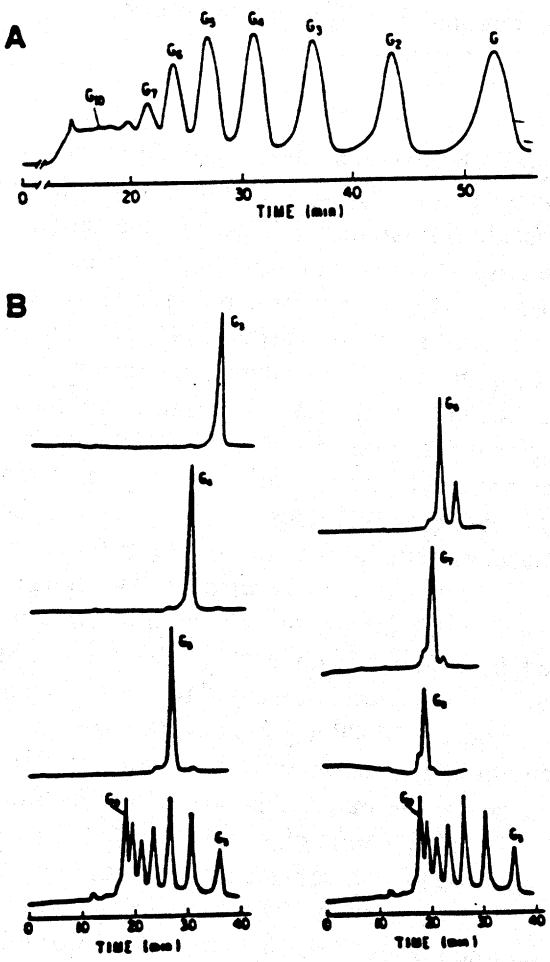

Fig. 9.1. (A) Preparative HPLC of cellodextrins as described in [97] on a 20 × 300 mm column packed with AG 50W-X4 (Ag^+ form) cation-exchange resin, eluted at 1.1 ml/min with water at 85°C. Pressure: 150 psi. Sample size: 125 mg. (B) Analysis of peaks collected in (A) using same column and conditions. Designation above peaks refers to size of oligosaccharides. G_4, for instance, stands for cellotetraose, dp 4. RI detection.

by size exclusion and reversed-phase mechanisms. Use of single and combined preparative HPLC methods for other complex oligosaccharides using methods developed prior to 1988 are reviewed in [4].

A very high resolution technique just recently being applied to preparative fractionation of complex neutral oligosaccharides is HPAE chromatography on pellicular phases (See Section 9.3.4). Hoffmann et al. [66] and Gruppen et al. [26] isolated a variety of oligosaccharides from wheat-flour arabinoxylan using an HPAEC column and an alkaline mobile phase. Fractions were neutralized immediately with molar HCl and were desalted with gel permeation and/or anion-exchange resins

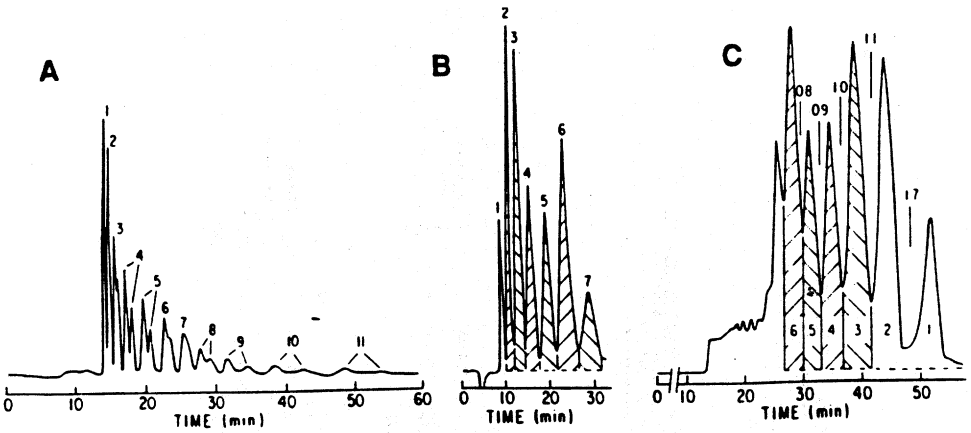

Fig. 9.2. Preparative HPLC of malto-oligosaccharides as described in [5]. (A) 12 mg injection on a 20 × 250 mm column packed with Dynamax C_{18} stationary phase eluted with water at 3 ml/min and 100 psi at room temperature. (B) 500 mg injection on a 20 × 250 mm column packed with aminopropyl silica gel eluted with acetonitrile–water (55:45) at 12 ml/min and 1000 psi at room temperature. (C) 125 mg injection on a 20 × 300 mm column packed with AG 50W-X4 (Ag^+ form) eluted at 1.1 ml/min with water at 85°C and at 150 psi. RI detection on all chromatograms. Numerals above peaks in (A) and (B) refer to oligosaccharide dp value. In (C), dp value is at base of peak and numeral at top refers to calculated resolution values (see Section 9.4.1).

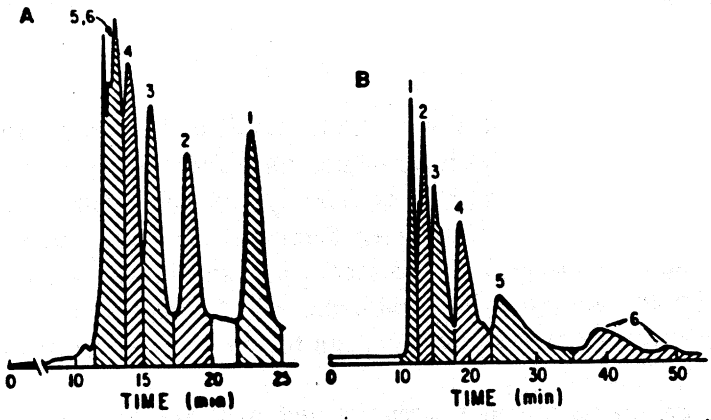

Fig. 9.3. Preparative HPLC of chitin-derived oligosaccharides as described in [51]. (A) 15 mg injection on a 7.8 × 30 mm column packed with HPX-87H cation exchange resin, eluted with 0.1 *N* H_2SO_4 at 0.3 ml/min and 75°C. (B) 140 mg injection on a 20 × 300 mm column packed with Dynamax C_{18}, eluted with water at 4 ml/min at room temperature. RI detection. Numerals above peaks refer to oligosaccharide dp value.

prior to ^{1}H-NMR spectroscopic analysis. Under these alkaline chromatographic conditions, these oligosaccharides, which contained β-1,4-linked D-xylopyranosyl groups, linked at positions 2 and 3, with α-D-arabinofuranosyl groups, were evidently very stable since nearly quantitative chromatographic recoveries were reported.

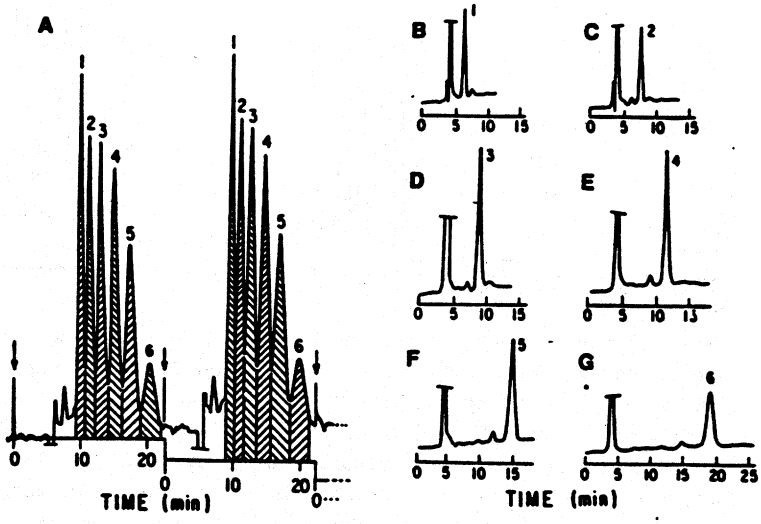

Fig. 9.4. Preparative HPLC of chitin oligosaccharides on an aminopropyl silica gel stationary phase using automated, repetitive injections as described in [51]. (A) Sequence of two automated injections (100 mg each) on a 20 × 250 mm column eluted at 12 ml/min with acetonitrile–water (60:40). (B–G) Purified oligosaccharides collected from (A). RI detection. Numerals above peaks refer to dp value of oligosaccharides.

Reddy and Bush [27] used HPAEC with dilute NaOH mobile phases to separate and isolate neutral oligosaccharides from human milk and neutral oligosaccharide alditols derived from mucin glycoproteins. The system employed a commercial anion micromembrane suppressor unit (see Section 9.2.7), attached in-line and after the PAD, to desalt fractions. The oligosaccharides and alditols were very stable under these conditions and were isolated with about 90% recovery from the system. The isolated oligosaccharides were pure enough for direct mass spectrometry and NMR spectroscopy.

Neutral, reduced oligosaccharides (alditols) such as those described above, are only weakly retained on HPAEC columns, resulting in poorer separations. Such alditols are relatively well retained on porous graphitized carbon columns [58], enabling several mg of alditol to be isolated from small (4.6 × 100 mm) columns with nearly complete (>90%) recovery.

9.5.4 Basic and acidic oligosaccharides

Few methods have been developed for basic oligosaccharides. Muraki et al. [104] used a combination of size exclusion and cation-exchange chromatography (see Section 9.3.3) to isolate pure oligosaccharides up to dp 8 from an enzymatic hydrolysis of chitosan.

Simple acidic oligosaccharides such as oligogalacturonic acids have been isolated on weak anion-exchange [64] and HPAEC phases [25] (see Section 9.3.4). The latter

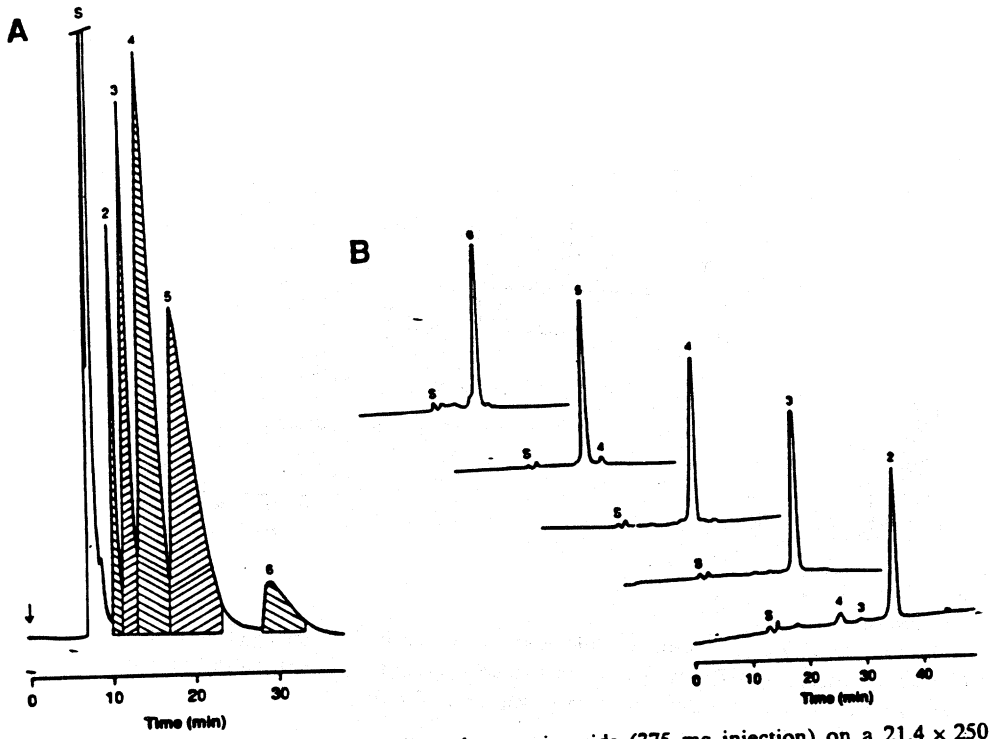

Fig. 9.5. (A) Preparative HPLC of oligogalacturonic acids (375 mg injection) on a 21.4 × 250 mm column packed with Dynamax NH_2 aminopropyl silica gel eluted at 10 ml/min with 0.9 M acetate buffer, pH 5. (B) Analysis of purity of oligosaccharides isolated in (A) on a HPX-22H analytical column eluted with.01 *N* H_2SO_4 at 0.4 ml/min. RI detection. Numerals over peaks refer to oligosaccharide dp values. See [64] for additional details.

phase has a low capacity but the former is useful for the gram-scale isolation of normal (Fig. 9.5) and unsaturated (Fig. 9.6) oligogalacturonic acids with purities of 95% or greater. Oligogalacturonic acids have also been fractionated on reversed-phase columns (see Section 9.3.2) when ion-pairing reagents have been added to the mobile phase [105,106]. Ion-pairing reversed-phase chromatography has also been used for the small-scale fractionation of acidic oligosaccharides derived from alginates [107] and carrageenans [106].

Sulfated oligosaccharides produced from the action of heparin lyase on heparin have been isolated [108] in mg quantities by HPLC on strong anion-exchange silica gels (see Section 9.3.4). Oligosaccharides from dp 2 through 6 were isolated.

Glucuronoxylo-oligosaccharides (10–100 mg quantities) derived from corn hemicelluloses were isolated on a semi-preparative amino-bonded silica gel using a mobile phase of acetonitrile/water/acetic acid [109].

Sialyl-oligosaccharides and alditols may be isolated by preparative HPLC on anion-exchange stationary phases [67,69,110]. Those derived from both *N*- and *O*-

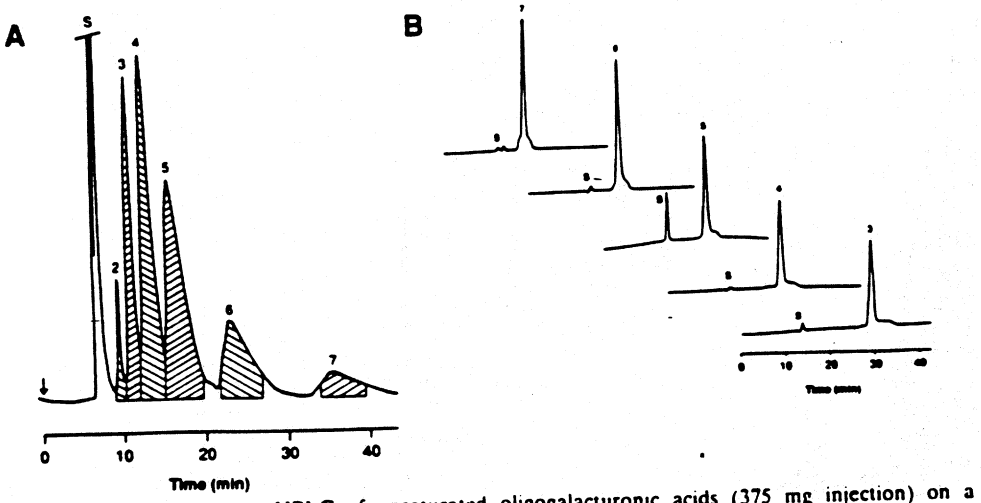

Fig. 9.6. (A) Preparative HPLC of unsaturated oligogalacturonic acids (375 mg injection) on a 21.4 x 250 mm column packed with Dynamax NH_2 aminopropyl silica gel. (B) Analysis of purity of oligogalacturonic acids isolated in (A). Other conditions identical to Fig. 9.5.

glycosylated glycoproteins [69] were purified on a high-performance anion-exchange phase usually used for protein purification. Oligosaccharides were eluted rapidly and with high resolution by a linear NaCl gradient. The resulting oligosaccharides were readily characterized by ^{1}H-NMR spectroscopy. For complete purification, sialylated oligosaccharides and alditols frequently require chromatography on several different phases [111] (and see [4] for a review). Klein et al. [110] used low pressure ion-exchange and size-exclusion chromatography to prepare fractions that were subsequently purified by preparative HPLC on a weak anion-exchange (polar amino-bonded silica gel) followed by reversed-phase chromatography. The use of volatile buffers with these two preparative HPLC steps facilitated the direct evaporation and subsequent NMR spectroscopic analysis of the purified sialylated oligosaccharide-alditols. Mawhinney et al. [112] also used several sequential liquid chromatographic techniques, including final HPLC on a weak anion-exchange (polar amino-bonded silica gel) stationary phase for purification of thirteen novel glycoprotein-derived oligosaccharides that contained both sulfate and sialyl groups.

Sialylated and other acidic oligosaccharides may be fractionated on reversed-phase columns when an ion-pairing reagent, usually an alkylamine, is added to the mobile phase. The ion-pairing reagent, which enhances retention and resolution of anionic oligosaccharides, can subsequently be removed from the fractions by cation-exchange resin treatment. Pre-fractionated, mono-sialylated oligosaccharides from human milk have been fractionated by this method [56] in sufficient quantity and quality for mass spectrometric and ^{1}H-NMR spectroscopic analysis.

Sialylated alditols derived from sialyllactose and di-sialyllacto-*N*-tetraose [58,59] are well retained on porous graphitized carbon phases (see Section 9.3.2). Their loading capacity is somewhat higher than that of most HPAEC phases. A major advantage of these stationary phases is their reported [59] ability to separate both sialylated and non-sialylated oligosaccharides in the same gradient elution. Unlike HPAEC phases which usually require elution with buffers containing non-volatile components, graphite-type phases can be used with aqueous mobile phases containing volatile trifluoroacetic acid. Oligosaccharide fractions isolated by this technique may be simply evaporated to remove trifluoroacetic acid prior to subsequent NMR spectroscopic or mass spectrometric analysis.

9.5.5 Glycopeptides and other glycoconjugates

Reversed-phase HPLC is frequently used for the isolation of glycopeptides (see [4], [113] and Chapter 2 for reviews). A two-step method, each using an octyl-bonded silica gel, was used for the multi-micromole isolation of highly purified asialo-triantennary glycopeptides from bovine fetuin [114]. Up to 75 mg of tryptic glycopeptides could be injected onto a 20 x 250 mm column. A similar system [115] was used for isolation of both N- and O-linked glycopeptides from tryptic digests of the same glycoprotein. Davies et al. [58] used a special porous graphitized carbon stationary phase (see Section 9.3.2), eluted with acetonitrile/aqueous trifluoroacetic acid for the separation of these glycopeptides. This relatively new graphite-type packing was reported to have unique characteristics, allowing good selectivity and capacity for glycopeptides containing relatively hydrophobic peptide sequences as well as those containing acidic amino acids and sialic acids.

9.6 BIBLIOGRAPHIC INFORMATION

Table 9.2 lists references to published methods for preparative HPLC of specific carbohydrates and derivatives. In a few instances where no actual references exist for preparative HPLC, references are given for published analytical-scale techniques that are readily scaled up.

TABLE 9.2

SELECTED PREPARATIVE HPLC TECHNIQUES FOR CARBOHYDRATES

Carbohydrate class	Specific example	Scale	Reference
Neutral monosaccharides and derivatives	Plant cell-wall sugars	1–100 mg	15,73
	Glucose, fructose	mg–kg	3,9,10,14,116,117
	2-Deoxy-2-fluoro-D-hexoses	2.5 g	118
	Inositols and inositol glycosides	mg	119,57
	Methyl- and benzyl glycosides	mg–g	74,75
Acidic or basic monosaccharides and derivatives	2-amino-2-deoxy-hexoses and derivatives	ng–mg	95,120,121,122
	Amadori products (1-amino-1-deoxy-2-fructose derivatives)	mg	96
	Nucleotide-diphosphoheptoses	mg	123
	Gallic acid glucosides	5–15 mg	76
	Sialic acids	μg–mg	94
	Sugar phosphates	ng–mg	83,84
	Inositol phosphates	ng–mg	87–93
	Uronic acids	mg	14,15
Neutral oligosaccharides	Lactulose, maltulose, cellobiulose, sucrose, trehalulose	100 mg	14,61,124
	Methyl maltosides and *O*-methylated sucrose derivatives	g	125,126
	Xylo-oligosaccharides	mg	52,127
	Isomalto-oligosaccharides	mg	52
	Malto-oligosacsaccharides	mg–g	5,6,44
	Cello-oligosaccharides	mg–g	52,97,128
	Chitin oligosaccharides	mg–g	51
	Inulin and other fructo-oligosaccharides	mg	101,102
	Arabinoxylan oligosaccharides	μg–mg	26,66
	Xyloglucan oligosaccharides	μg–mg	129–131
	Methylated cyclodextrins	mg–g	132,133
	Maltosyl-, Panosyl- and other branched cyclodextrins	g	134
	Cyclosophoroses	mg	135
	"Functionalized" dextrins	mg	55
	Branched gluco-oligosaccharides from glycosyltransferase/acceptor reactions	mg	136
	Galactose oligosaccharides prepared from transgalactosylation reaction of lactitol	mg	137
	Oligosaccharides and alditols derived from glycoproteins	μg–mg	20,27,58,59,71,121,138–144
	Human milk oligosaccharides	μg–mg	27,53,58,145,146
Basic oligosaccharides	Glucosamino oligosaccharides	mg	104

TABLE 9.2 (continued)

Carbohydrate class	Specific example	Scale	Reference
Acidic oligosaccharides	Hyaluronan tetra- and hexasaccharides	mg	147
	p-Coumarate or ferulate esters of plant-derived oligosaccharides	mg	148
	Glucuronoxylo-oligosaccharides from hemicellulose	10–100 mg	109
	Oligogalacturonic acids	mg–gm	21,64,25,97
	Sialyl-oligosacarides and alditols from N- and O-linked glycoproteins, colostrum or milk	μg–mg	56,58,67,69,110, 149–155
	Sulfated oligosaccharides from glycosaminoglycans	μg–mg	68,108,156,157
	Phosphorylated oligosaccharides from glycoproteins	mg	151,153
	Sulfated sialyl-oligosaccharides	mg	112
Glycopeptides	N- and O-Linked glycopeptides	μg–mg	58,59,158–161, 114,115

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